E. 3 Cloning of the final construct

In order to express the soluble Methanemonooxygenase (sMMO) functionally in *E. coli* all genes for its subunits have to be assembled in one construct. Therefore, all mmo genes are cloned together with a RBS. Then the single genes are cloned together in pairs of two. Two of these pairs are again assembled together and after one last cloning step, the construct is complete.

02.07.2014

Restriction of vector and fragments

- ➔ Plasmid 2 (RBS) with SpeI and PstI --- 10 µg digested with 0,5 µl enzyme
- ➔ Plasmid 136 (mmoB)
- ➔ Plasmid 147 (mmoC)
- ➔ Plasmid 137 (mmoD)
- ➔ Plasmid 148 (mmoX)
- ➔ Plasmid 149 (mmoY)
- ➔ Plasmid 150 (mmoZ)

} 10 µg digested with 0,5 µl enzyme

Gelelectrophoresis

-gelelctrophoresis in 1,5 % agarose gel at 140 V

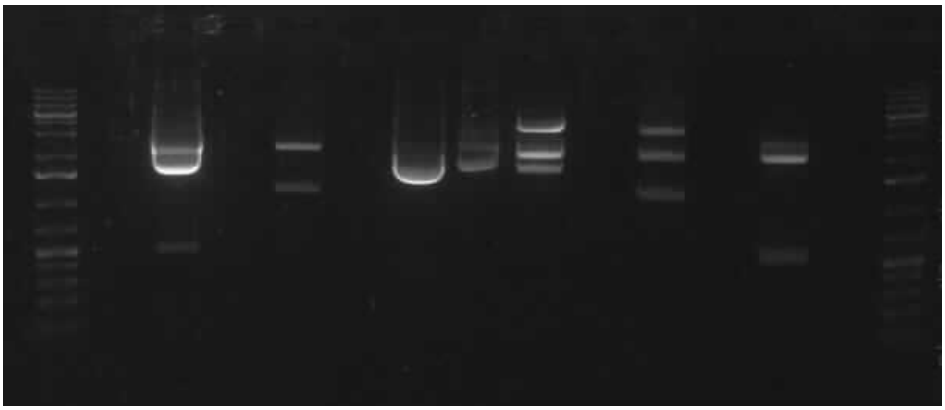


Fig. 3.1 *Gel-purification of mmo-plasmid-restriction – **1**: mmoB (Exp. lenght:426); **2**: mmoC (Exp. lenght: 1047); **3**: mmoD (Exp. lenght: 312); **4**: mmoX (Exp. lenght: 1584); **5**: mmoY (Exp. lenght: 1170); **6**: mmoZ (Exp. lenght: 513) **M**: 1kb GeneRuler plus*

- ➔ Band are not strong enough. More DNA-material is needed.
  Therefore, inoculation of 100 ml over night cultures of mmoC (GS 53), mmoX (GS 48), mmoY (GS 44) and mmoZ (GS 17).

03.07.2014

Oli

Midi-Prep

➔ of over night cultures of mmoc, mmoX, mmoY and mmoZ.

Restriction

➔ of mmoB (plasmid 136), mmoC (plasmid 151), mmoD (plasmid 137), mmoX (plasmid 152), mmoY (plasmid 153) and mmoZ (plasmid 154) with 1µl XbaI and 1µl PstI

➔ RBS-vector (plasmid 6) with 1µl SpeI and 1µl PstI

	mmoB	mmoC	mmoD	mmoX	mmoY	mmoZ	RBS
Plasmid	26µl	25µl	15µl	35µl	8µl	9µl	20µl
Cutsmart	6µl	6µl	6µl	6µl	6µl	6µl	6µl
XbaI-HF	1µl	1µl	1µl	1µl	1µl	1µl	-
SpeI-HF	-	-	-	-	-	-	1µl
PstI-HF	1µl	1µl	1µl	1µl	1µl	1µl	1µl
Phosphatase	-	-	-	-	-	-	1µl
H2O	16µl	17µl	27µl	7µl	34µl	33µl	21µl
Sum	60µl	60µl	60µl	60µl	60µl	60µl	60µl

➔ Restriction incubated at 37 °C over night (16 hours)

04.07.2014

Oli. Zen-Zen, Carsten

Purification via 1,5% agarose gel electrophoresis:

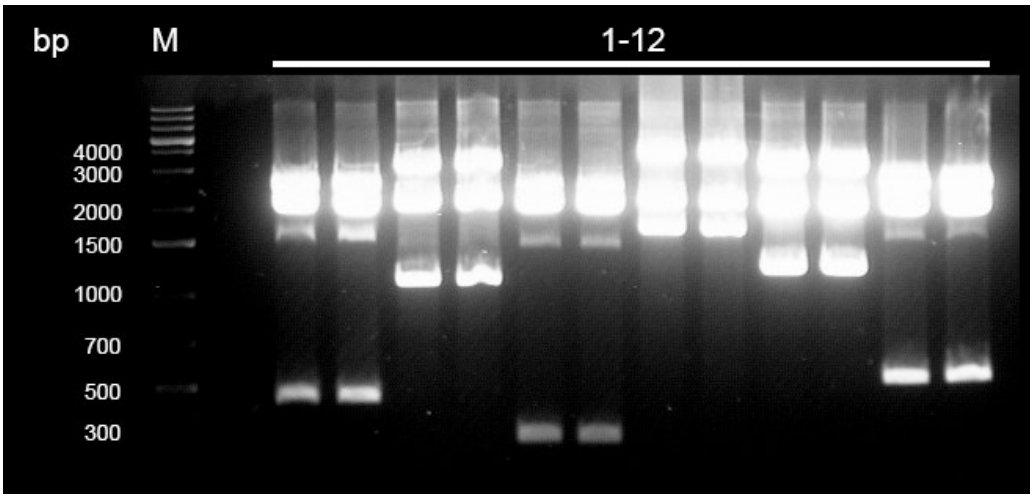


Fig. 3.2 *Repeat of mmo-plasmid-restriction – **1-2**: mmoB (Exp. lenght:426); **3-4**: mmoC (Exp. lenght: 1047); **5-6**: mmoD (Exp. lenght: 312); **7-8**: mmoX (Exp. lenght: 1584); **9-10**: mmoY (Exp. lenght: 1170); **11-12**: mmoZ (Exp. lenght: 513)*

→ apparently the used PstI enyzm is not a HF enzyme, therefore its efficiency in cutsmart puffer is only 50%! In further experiments a sequential restriction will be performed.

Ligation of all six fragments into restricted RBS-vector

mmo fragment	7 µL
Backbone	2 µL
T4 DNA Ligase	0,5 µL
T4 Ligase Buffer	1 µL
∑	**10,5 µL**

Transformation into competent cells and platting.

05.07.2014

Steffen, Carsten, Oli

No colonies on any plate. It might be that the DNA after restriction was defiled. Therefore the remaining of the restriction is purified again.

Ligation is repeated:

mmo fragment	14 µL
Backbone	5 µL
T4 DNA Ligase	1 µL
T4 Ligase Buffer	2 µL
∑	**22 µL**

Ligation is performed over night at RT.

06.07.2014

Carsten,Oli

Transformation of ligation and plating. -> incubation at 37°

07.07.2014

Oli

All plates are empty… Due to that, the whole construction is restarted.

All plasmids of mmo genes (plasmid 136, 137, 151- 154) are restricted with XbaI and the RBS-vector (pSB1C3-backbone) with SpeI for 3 hours. Afterwards the NEB buffer 3.1 is added to all reactions and restricted with PstI over night.

Used amount of DNA: mmo genes 10 µg each and vector 15 µg.

1,5% agarose gel electrophoresis with 100V:

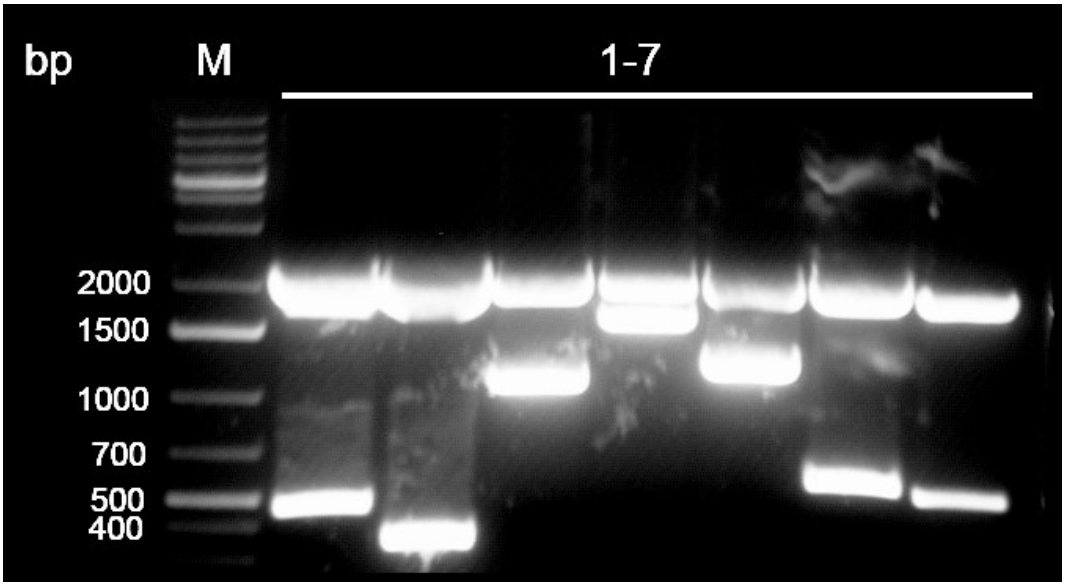


Fig. 3.3 Gel-purification of mmo-plasmid-restriction – 1: mmoB (Exp. lenght:426); 2: mmoC (Exp. lenght: 1047); 3: mmoD (Exp. lenght: 312); 4: mmoX (Exp. lenght: 1584); 5: mmoY (Exp. lenght: 1170); 6: mmoZ (Exp. lenght: 513)

→ restriction worked this time

08.07.2014

Oli

Gel elution of positive band from yesterday.

Ligation (for 2hours at RT) and transformation of all mmo genes into RBS vector (7 µL insert + 2 µL vector; concentration unknown).

09.07.2014

Oli

All transformation plates are showing colonies.

Colony-PCR to screen for positive clones (primer 83 and 84 – binding on prefix and suffix region)

1% agarose gel electrophoresis at 140V:

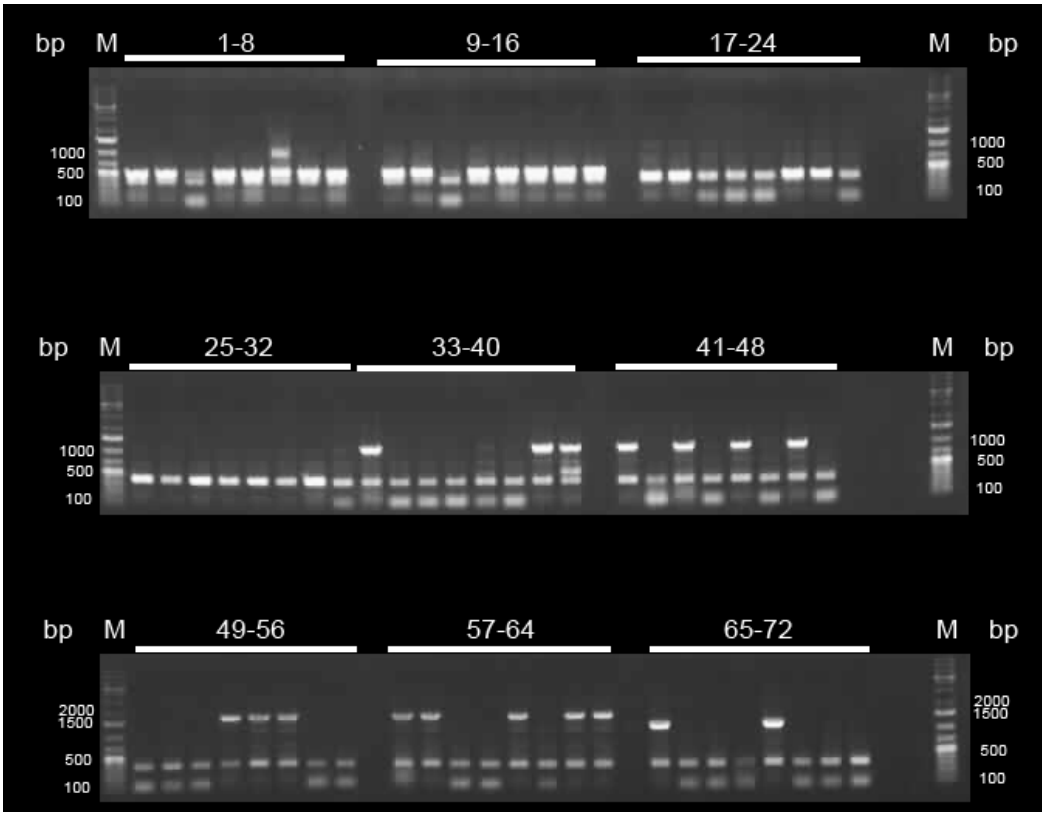


Fig. 3.4 *Colony-PCR –* ***1-16****: RBS-mmoB;* ***17-32****: RBS-mmoC;* ***33-48****: RBS-mmoD;* ***49-64****: RBS-mmoX;* ***65-72****: RBS-mmoY*

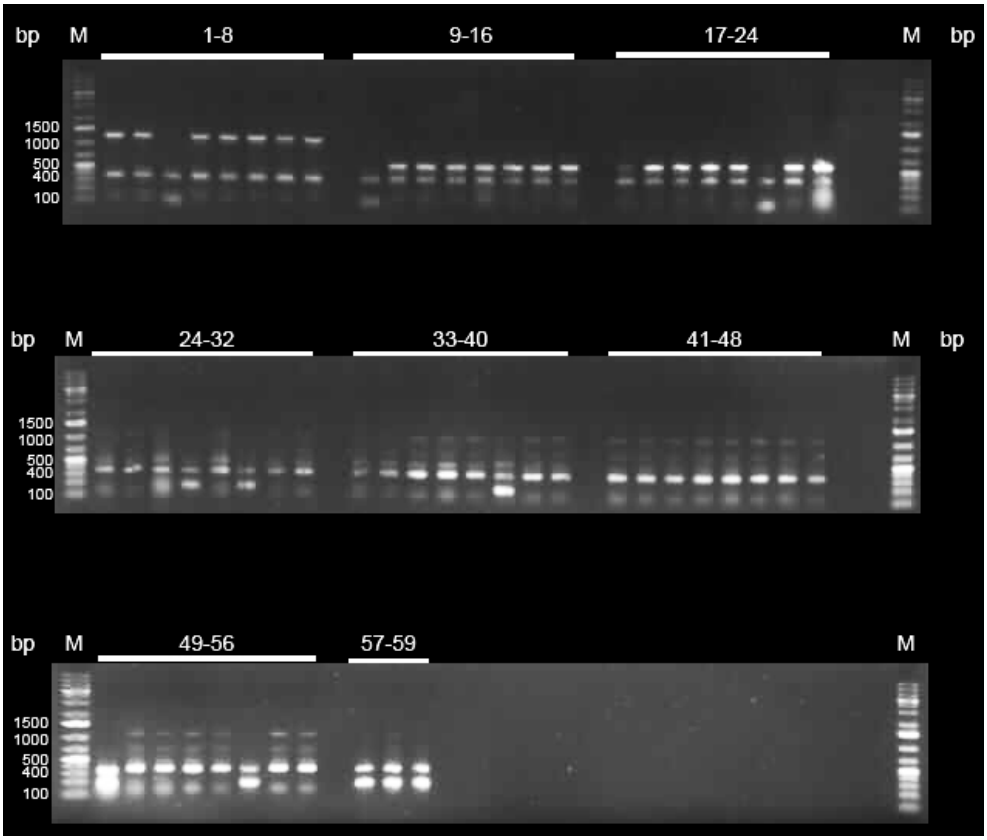


***Fig. 3.5** Colony-PCR – **1-8**: RBS-mmoY; **9-24**: RBS-mmoZ; **25-40**: MeOH-Promotor (long); **41-56**: MeOH-Promotor (short); **57-59**: GUS*

- ➔ All ligations have at least 2 positive clones, except GUS.
- ➔ 2 colonies of each ligation are picked for over night cultures.

10.07.2014

Oli

Plasmid preparation of all over night cultures.

As an additional control, 2 µg of the isolated DNA was test digested with NotI, to double check for correct insert length.

1,5% agarose gel electrophoresis at 140V:

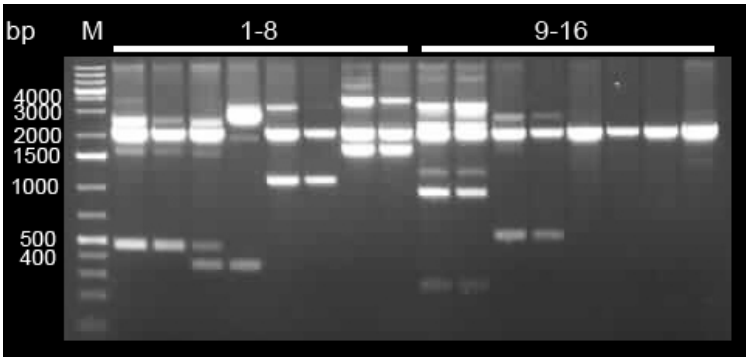


Fig. 3.6 ***1-2****: mmoB (Exp. lenght:426);* ***3-4:*** *mmoD (Exp. lenght: 312);* ***5-6****: mmoC (Exp. lenght: 1047);* ***7-8****: mmoX (Exp. lenght: 1584);* ***9-10****: mmoY (Exp. lenght: 1170);* ***11-12****: mmoZ (Exp. lenght: 513);* ***13-14****: MeOH-Promotor (long) (Exp. lenght: 530);* ***15-16****: MeOH-Promotor (short) (Exp. lenght: 347)*

→ clones 1-2 of mmoB, 3-4 of mmoD, 5-6 of mmoC, 7-8 of mmoX, 9-10 of mmoY, 11-12 of mmoZ are positive

→ clones 1,4,5,7,9 and 11 are used for further experiments

→ no positive for methanol promoter

Restriction of positive clones. Inserts (mmoC, mmoY and mmoZ) with XbaI and PstI and vectors (mmoX, mmoB and mmoD) with SpeI and PstI. First restriction with XbaI/SpeI for 3h afterwards restriction with PstI over night.

11.07.2014

Oli

<u>Gel-Purification</u>

➔ over 1,5% agarose gel electrophoresis with 100V:

<u>Ligation</u>

➔ of mmoX+mmoY, mmoB + mmoZ and mmod + mmoC

<u>Transformation</u>

Of the mmoXY, mmoBZ and mmoDC constructs (100 µl and rest-plates)

12.07.2014

Carsten, Maren

<u>Colony-PCR</u>

Colony-PCR of 32 colonies of each transformed construct from 11.07.2014 (mmoXY, mmoBZ, mmoDC) with Go-Taq protocol.

1,0% agarose gel electrophoresis with 140V:

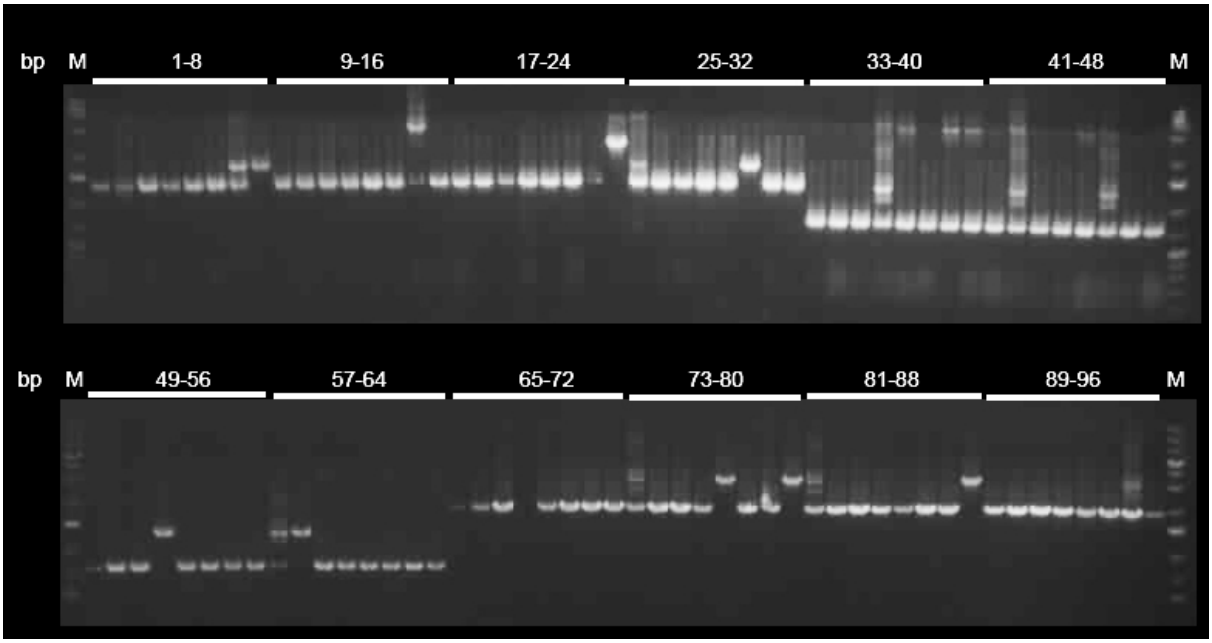


Fig. 3.7 Colony-PCR – 1-32: mmoDC; 33-64: mmoBZ; 65-80: mmoBZ; 81-96: mmoXY

13.07.2014

Melanie

Overnight culture

➔ Of clones 40+42 (mmoCD), 84+90 (mmoBZ) and 109+112 (mmoXY) in 5 ml 2YT-medium plus Chloramphenicol

14.07.2014

Oli, Anna, Carsten

Positive results for all samples send for sequencing on 10.07.2014!

Plasmid preparation of all over night cultures.

➔ DNA concentrations are too low, therefore inoculation of over night cultures of the same clones again (1x 5 mL 2YT-Ca-medium)

15.07.2014

Nils, Oli

Plasmid preparation of over night cultures.

Restriction with NotI for 3h to test the fragments for correct size

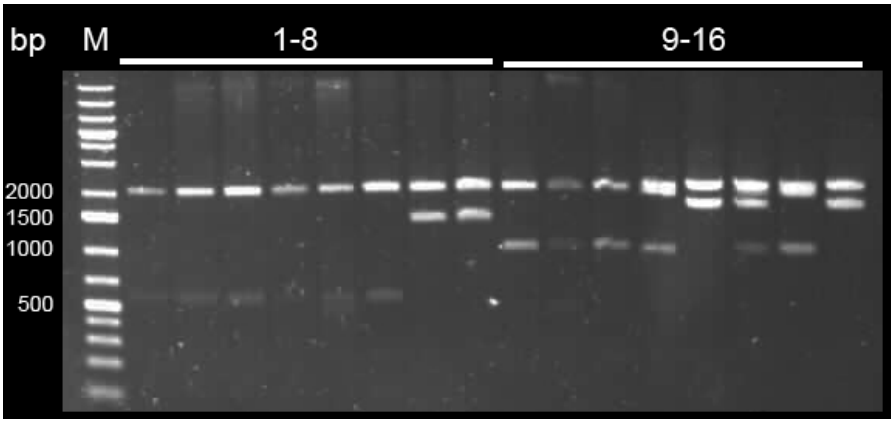


*Fig. 3.8 Test-restriction with Not1 (after 45 mins) – **1-6**: Lac-RBS-mmoD-HIS-TT; **7-8**: mmoDC; **9-11**: mmoBZ; **12-16**: mmoXY*

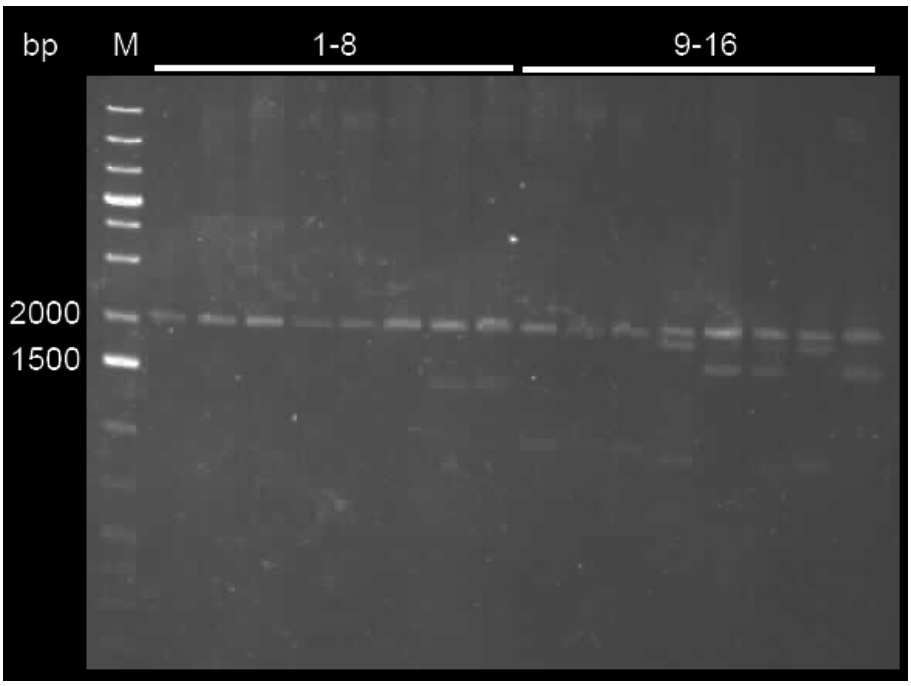


*Fig. 3.9 Test-restriction with Not1 (after 85 mins) – **1-6**: Lac-RBS-mmoD-HIS-TT (exp. lenght 572); **7-8**: mmoDC (exp. lenght1472 ; **9-11**: mmoBZ (exp. lenght 1007); **12-16**: mmoXY (exp. lenght 2046+1877+945)*

→ band 1-3 of Lac-RBS-mmoD-His-TT (clone 8, 12, 18); mmoCD clone 40 and 62 are correct (band 40, 62); mmoBZ clone 84 and 90 are correct (band 9, 11); mmoXY clone 109 and 120 are correct (band 109, 120)

➔ All positive clones are send for sequencing.

Overnight culture of two correct clones of each construct 3x5 ml 2xYT-Ca G

16.07.2014

Sequential Restriction of double constructs XY, BZ and DC

Fragment: **XY clone 109**	20 µL (=10µg)
Cut-Smart Buffer	3 µL
SpeI	2 µL
H_2O	5 µL
∑	**30 µL (incubation for 3h at 37°C)**
+ H_2O	4 µL
+ PstI	2 µL
+ Puffer 3.1	4 µL
∑	**40 µL (incubation over night at 37°C)**

Fragment: **BZ clone 84**	20 µL (=10µg)
Cut-Smart Buffer	3 µL
XbaI	2 µL
H_2O	5 µL
∑	**30 µL (incubation for 3h at 37°C)**
+ H_2O	4 µL
+ PstI	2 µL
+ Puffer 3.1	4 µL
∑	**40 µL (incubation over night at 37°C)**

Fragment: **DC clone 40**	20 µL (=10µg)
Cut-Smart Buffer	3 µL
EcoRI and SpeI	2 µL each
H_2O	3 µL
∑	**30 µL (incubation for 3h at 37°C)**

Fragment: **TT**	20 µL (=10µg)
Cut-Smart Buffer	3 µL
EcoRI and XbaI	2 µL each
H_2O	3 µL
∑	**30 µL (incubation for 3h at 37°C)**

17.07.2014

Christian, Oli

1,5% gel electrophoresis at 100V for 90 min

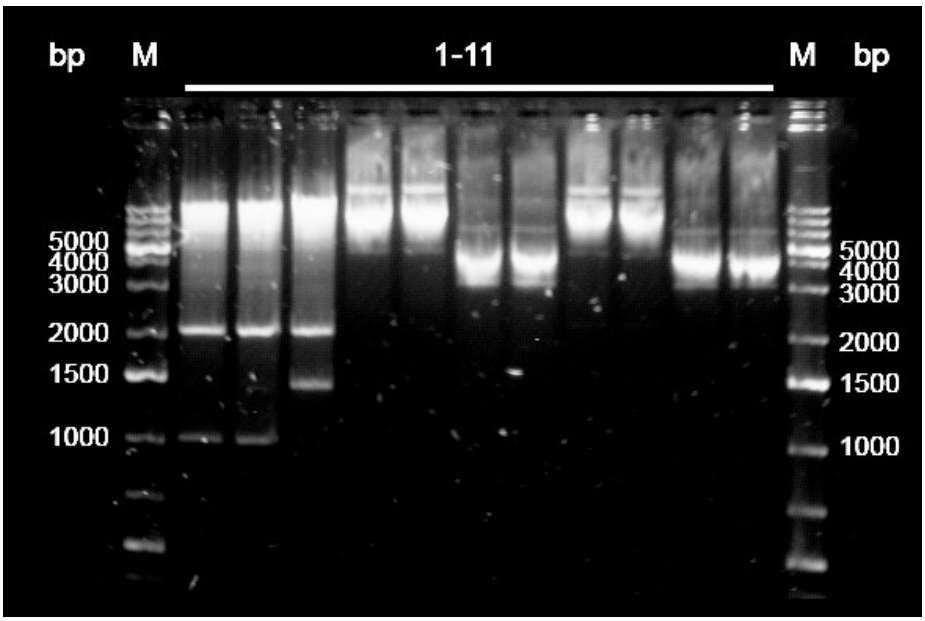


Fig. 3.10 *Gel-purification of double-constructs – **1-2**: mmoBZ; **3**: mmoDC*

➔ Bands are way to weak. Additionally is looks like there is a lot genomic DNA in the sample. Maybe something is wrong with the plasmid preparation. To check this a 1% agarose gel with the plasmid preparation is run.

17.7 1% agarose gel at 100V ca 1h Test-Gel due to genomic DNA see above

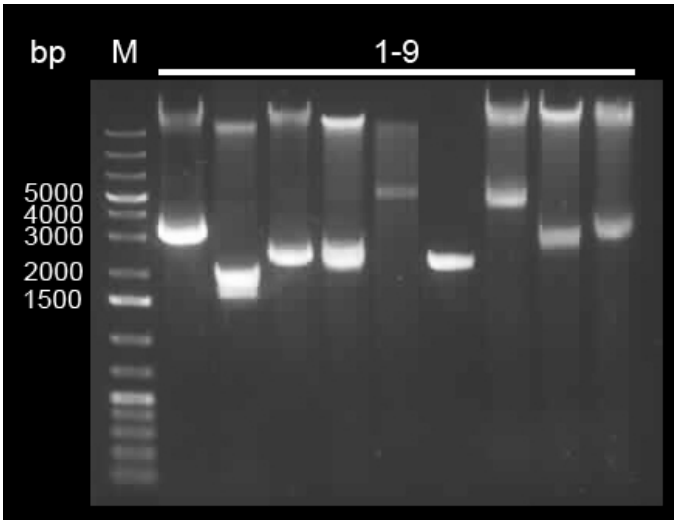


Fig. 3.11 *Test-restriction –* ***5:*** *mmoXY;* ***6****: TT [B0015];* ***7****: mmoXY;* ***8****: mmoBZ;* ***9****: mmoDC*

Over night cultures for medi-preps of:

18.07.2014

Anna, Melanie, Christian, Steffen

Medi-prep of overnight cultures.

Restriction of XY (with PstI and SpeI), BZ (with XbaI and PstI), DC (with EcoRI and SpeI) and TT-Vector (with EcoRI and XbaI). Purification over gel.

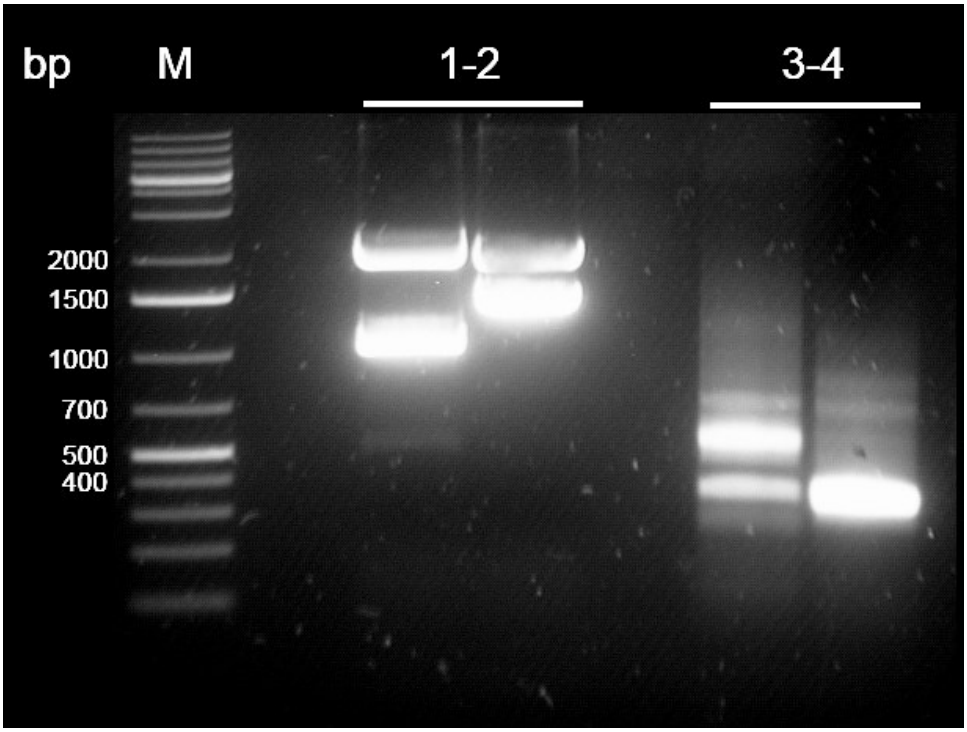


Fig. 3.12 *Gel-purification – **1**: mmoBZ; **2**: mmoDC;*

➔ All bands show the expected length! Gel elution, Ligation of BZ + XY and DC + TT-vector and transformation.

19.07.2014

Christian, Oli

Plasmid preparation of over night cultures and preparation of glycerin stocks of all five positive clones from yesterday.

Colony PCR of the end-fragments BZ+XY and DC+TT. M1 and M2was preformed

48 clones of fragment BZ+XY and 48 clones of fragment DC+TT

Things To be done on 20th of July

Gelelectrophoises of Col PCR-> inoculation of positive clones

20.7.2014

Christian

BZ+XY + overhang Primer ca 4000 bp; DC+TT+ overhang ca 1900 bp

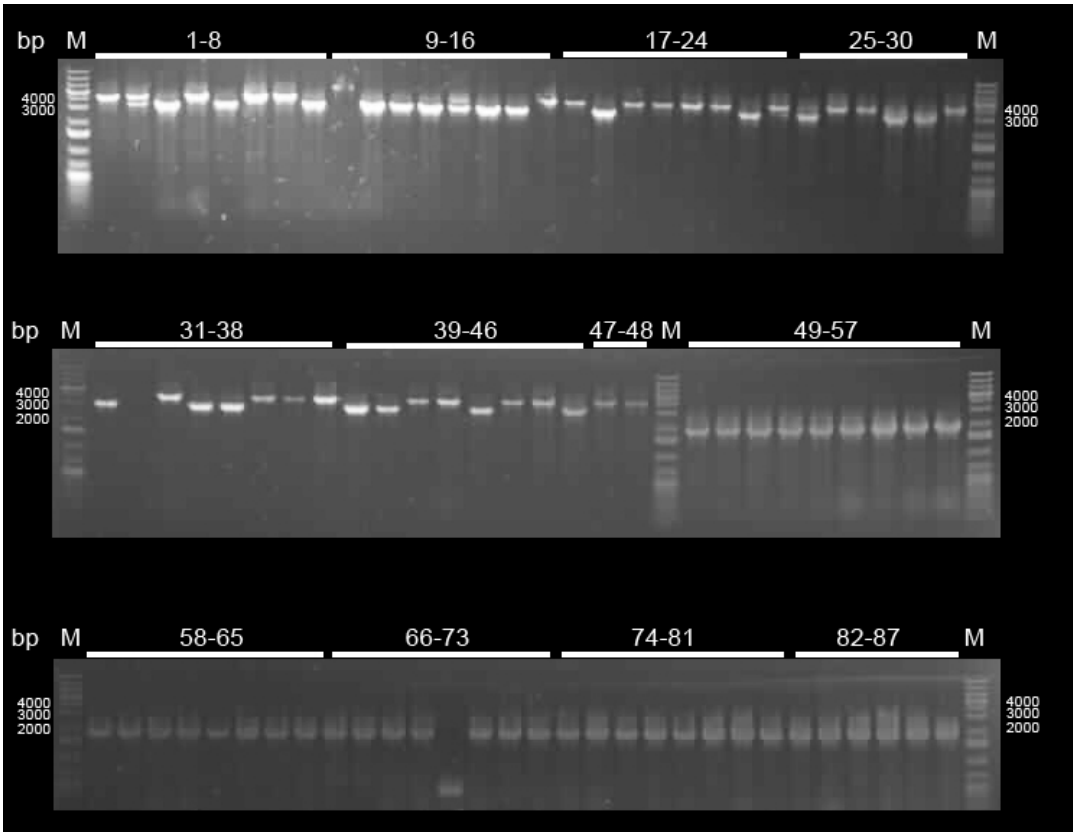


Fig. 3.13 *Colony-PCR – **1-48**: mmoBZXY; **49-87**: mmoDC + TT*

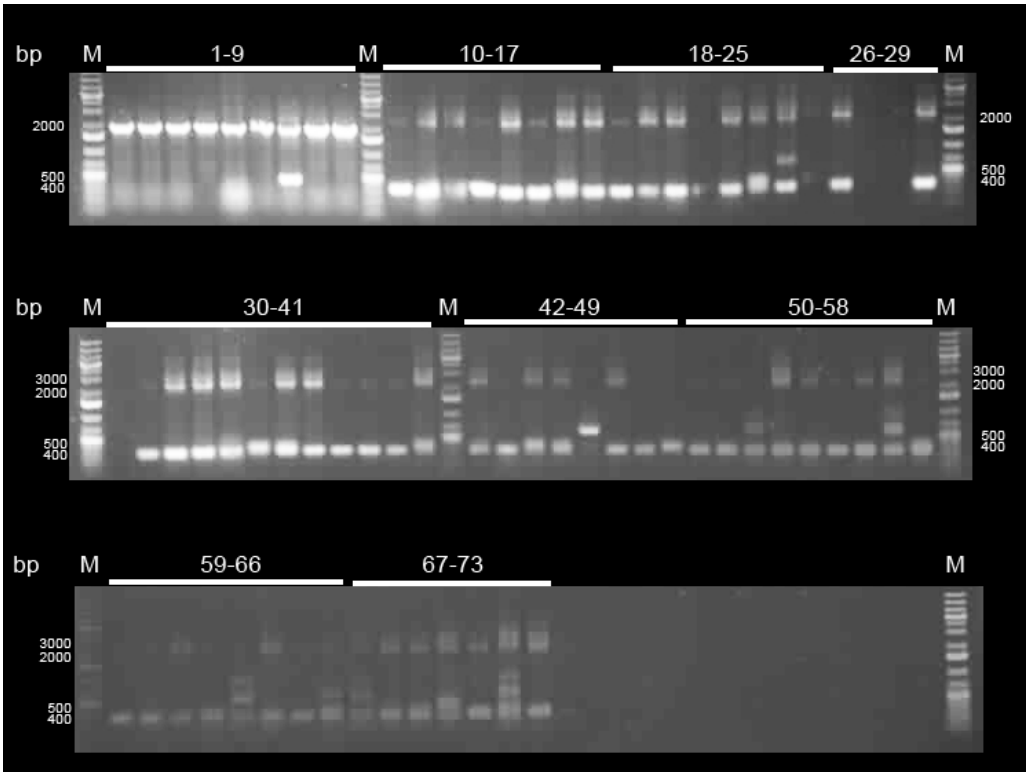


Fig. 3.14 *Colony-PCR – **1-9:** mmoDC + TT; **59-73**: mmoDC + TT*

Overnight cultures of positive clones were prepared ; BZ+XY (masterplate clone number: 1,4,6 and 17 (gel band 2,5,7,18)

DC+TT(masterplate clone number 40,41,42,48) ; M1 masterplate clonenumber 15 M2 masterplate clone number 5,22)

Masterplates are stored in the fridge (Date 19.7)

21.07.2014

Christian, Oli, Carsten

Plasmid preparation of over night cultures.

Test restriction with NotI of all preparations.

1,5% agarose gel electrophoresis at 120 V for 1 h:

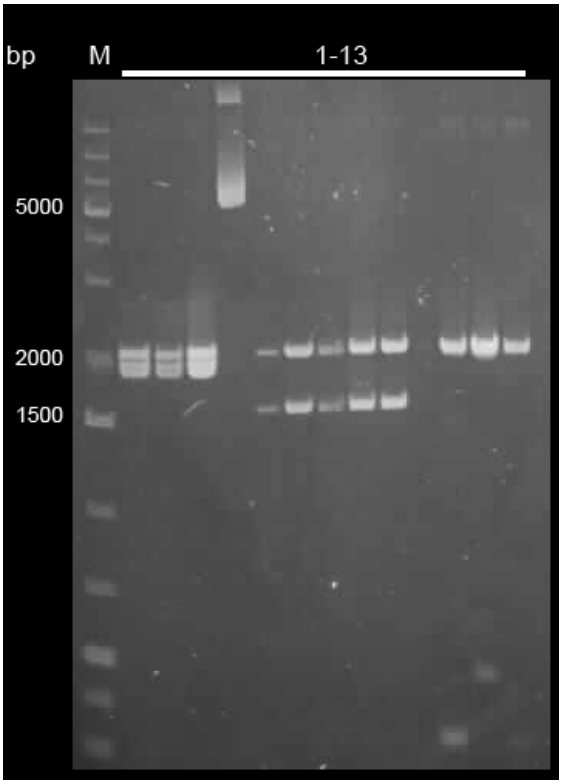


Fig. 3.15 *Test-restriction with Not1 – **1-4**: mmoBZXY (Clones 1,4,6,17) (Exp. lenght: 1877+1936+2040); **6-9**: mmoDC-TT (Exp. lenght: 2046)*

The fragments BZXY and DC-TT seem to be correct. Methanol-Promoters: Incorrect ordner? -> Will be sequenced! Clone 17 of BZXY seems to be wrong restricted.

Overnight culture for Midi-Prep

Clone 1 of BZXY and clone 40 of DC-TT are used for 100 ml overnight culture

22.07.2014

Oli, Anna, Carsten

Midi Prep

BZXY- and DC-TT-constructs: BZXY (2070,91 ng/µl) and DC-TT (1107,59 ng/µl)

Restriction for last cloning step (for both fragments in the promotor-vector at once)

1st restriction (2 h at 37 °C)		**2nd restriction (overnight at 37 °C)**
BZXY (plasmid 178)	4,83 µl	Only 1st restriction is neccessary
EcoRI HF	1 µl	
SpeI HF	1 µl	
Cutsmart	1 µl	

H2O	2,2 µl		
DC-TT (plasmid 179)	9,0 µl	PstI	1 µl
XbaI	1 µl	NIB 3.1 buffer	1,75
Cutsmart	1,5 µl		
H2O	3,5 µl		
Prom J23112 (plasm 3)	21 µl	PstI	1 µl
EcoRI HF	1 µl	NIB 3.1 buffer	2,9 µl
Cutsmart	2,6 µl		
H2O	1,5 µl		
Prom J23100 (plasm 4)	18,2 µl	PstI	1 µl
EcoRI HF	1 µl	NIB 3.1 buffer	2,5 µl
Cutsmart	2,2 µl		
H20	0,6 µl		

Restriction for last cloning step (for first combination of BZXY + DCTT and then in vector)

1st restriction (2 h at 37 °C)		**2nd restriction (overnight at 37 °C)**	
BZXY (plasmid 178)	4,83 µl	PstI	1 µl
SpeI	1 µl	NIB 3.1 buffer	1,3 µl
Cutsmart	1 µl		
H2O	2,2 µl		
DC-TT (plasmid 179)	9 µl	PstI	1 µl
XbaI	1 µl	NIB 3.1 buffer	1,75 µl
Cutsmart	1,5 µl		
H2O	3,5 µl		

23.07.2014

Rüdiger, Nils, Christian

Purification of cut fragments from the day before

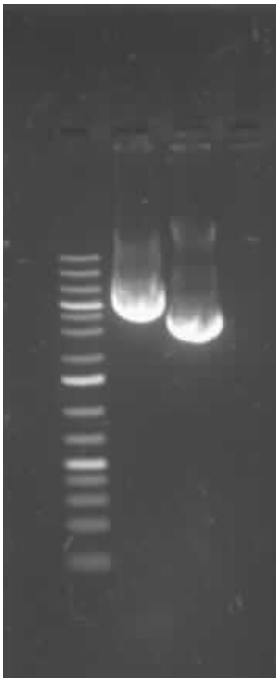
Vectors (Promotors) purificated with column, inserts (BZXY, DC-TT) with 1% agarose gel

Gelelution:

→ no marker (should be at the right) and no defined bands could be observed, gel is not used for elution and the restriction is repeated

Testing of integrity of PL 178 and 179

Gelelectrophoresis in 1 % agarose gel at 130 V



Fragment	M	PL 178	PL 179
Expected length [bp]	1 kb plus ladder	supercoiled	supercoiled
Band-No.	1	2	3
Positive		+	+

***Fig. 3. 16** – Testing of integrity*

Repetition of restriction

PL 178 (XYBZ)	4,83 µL
Cut-Smart Buffer	1 µL
Res 1 (e.g. EcoRI-HF)	1 µL
Res 2 (e.g. XbaI)	1 µL
H_2O	2,2 µL
∑	10 µL

PL 179 (DC-TT)	18 µL
1.	
Cut-Smart Buffer	3 µL
Res 1 (e.g. EcoRI-HF)	1,5 µL
H_2O	7,5 µL
2.	
PstI	1,5 µL
NEB 3.1 Buffer	3,5 µL
H_2O	0,5 µL
∑	35,5 µL

Gelelution

Bands were cut out and put into the freezer

24.04.2014

Melanie, Christian, Oli

Gel elution of both samples from yesterday were finished.GEM2014

→ Pl178 (XYBZ): 73,5 ng/µL PL179 (DC-TT): 113,8 ng/µL

Ligation end 1 : plasmid 3 J23112 (2µl) +plasmid 178 XYBZ (7µl Res E+S)+plasmid 179 DCTT (7µl res. X-P); 20µl sample (AmpR!)

Ligation end 2 : plasmid 4 J23100 (2µl) +plasmid 178 XYBZ (7µl Res E+S)+plasmid 179 DCTT (7µl res. X-P), 20 µl sample (AmpR!)
Ligation end 3 : plasmid 178 (2µl Res. S+P) + plasmid 179 DCTT (7µl res. X-P) 10 µl sample

Ligation for 1 hour at RT.

Transformation into competent E. coli cells and plating on agar plates. Incubation over night at 37°C.

Overnight culture of XY+BZ clone 1 and DC-TT clone 40 was inoculated (volume 15ml)

25.07.2014

Oli

Glycerin stock of all three over night cultures. Additionally a mini prep of those samples is performed.

Transformation of the final ligation showed colonies on plate 1 (J23112+XYBZDC-TT with RBS in between) and 3. Plate 2, which is similar to plate 1 except that it is a different promoter, did not show any colonies. Probably the constitutive promoter was to strong…?

-> Colony-PCR of plate 1 and 3, 48 colonies each.

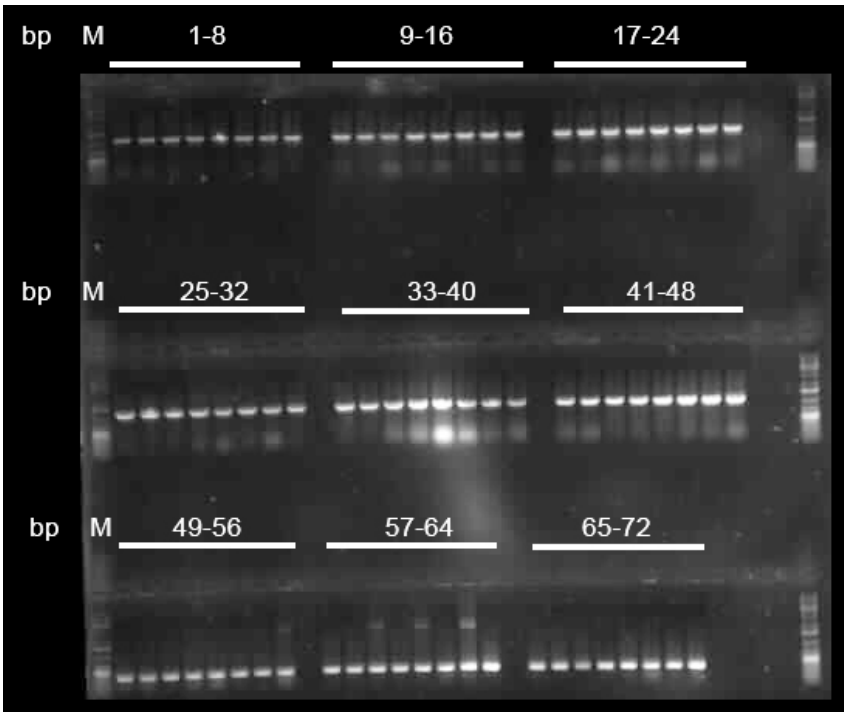


Fig. 3.17 Colony PCR – **1-48:** J23112-XYBZDC-TT **49-72:** XYBZDC-TT

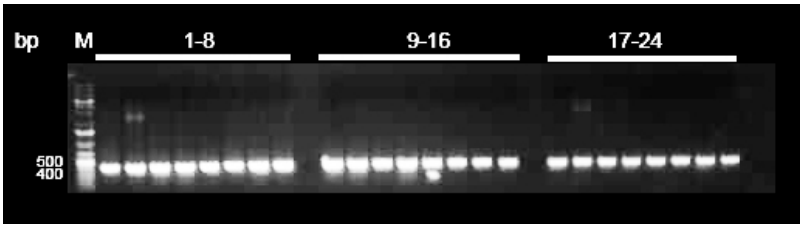


Fig. 3.18 Colony PCR – **1-24:** XYBZDC-TT

26.07.2014

Carsten

Mini Prep

➔ Not possible due to no grown overnight cultures (wrong antibiotic? Promotor vectors have Amp-resistence!)

28.07.2014

Melanie

Inoculation of over night cultures of all positive clones from 25.07.2014.

Clones: XYBZ+DC-TT 8, 11, 13, 15

29.07.2014

Oli

Plasmid preparation of all over night cultures.

All four samples are send for sequencing with VR primer (primer 89).

Steffen, Anna

Restriction of XYBZDCTT Klon 15 (Plasmid 195) with X+P and promoters J23112 and J23100 (Plasmid 3 and 4) with S + P

Plasmid-DNA	30 µL

Cut-Smart Buffer	6 µL
Res 1 (SpeI) for vector	1 µL
Res 2 (XbaI) for insert	1 µL
H_2O	15 µL
∑	53 µL

Step 1 -> Incubation for 1,5 h at 37°C Step 2 -> add Pst and NEB3 incubation over night at 37°C

Res 3 (Pst)	1 µL
NEB3	6 µL
∑	60 µL

30.07.2014

Nils, Niels, Steffen, Carsten

Purification of restriction

Vectors (J23100 and J23112) are purificated using Promega Wizard Kit column and insert (XYBZDCTT) by gel purification (1% agarose, 40 min at 120V).

Restriction of XYBZDCTT Klon 8 (192), Klon 11 (193), Klon 13 (194) and Klon 15 (Plasmid 195) with X+P

Step 1 -> Incubation for 1,5 h at 37°C

	Klon 8 (192)	Klon 11 (193)	Klon 13 (194)	Klon 15 (195)
Plasmid-DNA	30 µL	30 µL	30 µL	30 µL
Cut-Smart Buffer	5 µL	5 µL	5 µL	5 µL
Res 2 (XbaI) for insert	1 µL	1 µL	1 µL	1 µL
H_2O	14 µL	14 µL	14 µL	14 µL
∑	50 µL	50 µL	50 µL	50 µL

Step 2 -> add Pst and NEB3 incubATION over night at 37°C

Res 3 (Pst)	1 µL
NEB3	6 µL
∑	60 µL

31.07.2014

Steffen, Niels, Melanie

Gelextraction of restricted final constructs:

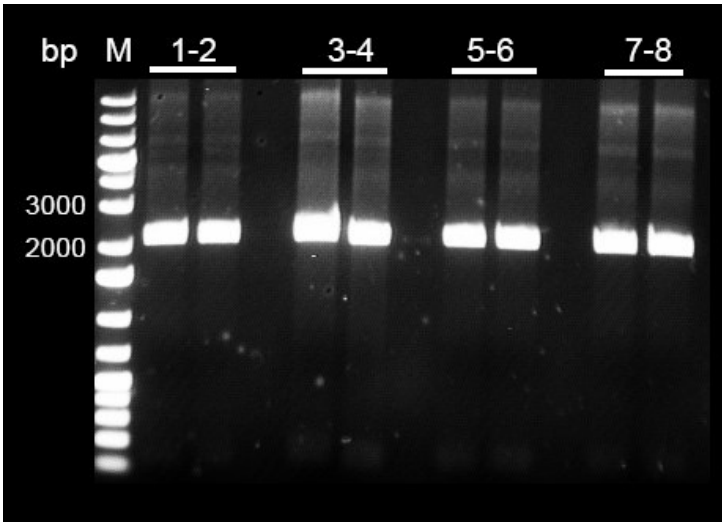


Fig. 3. 19 Gel-Purification – 1-8: Endconstruct mmoXYBZDC+TT (Exp. Length 5100 bp)

→ Restriction failed!

Sequencing results: **all four clones are negative.** That explains the false results of the last experiments. -> the last restriction step is repeated.

Plasmid 181 (XYBZ) and 182 (DC-TT)

Step 1 -> Incubation for 1,5 h at 37°C

Plasmid-DNA (~10µg)	20 µL
Cut-Smart Buffer	3 µL
Res 1 (SpeI) for vector	1 µL
Res 2 (XbaI) for insert	1 µL
H_2O	6 µL
∑	30 µL

Step 2 -> add Pst and NEB3 incubation over night

Res 3 (Pst)	1 µL
NEB 3.1 buffer	4 µL
H_2O	5 µL
∑	40 µL

01.08.2014

Niels, Oli

1,0 % agarose gel electrophoresis

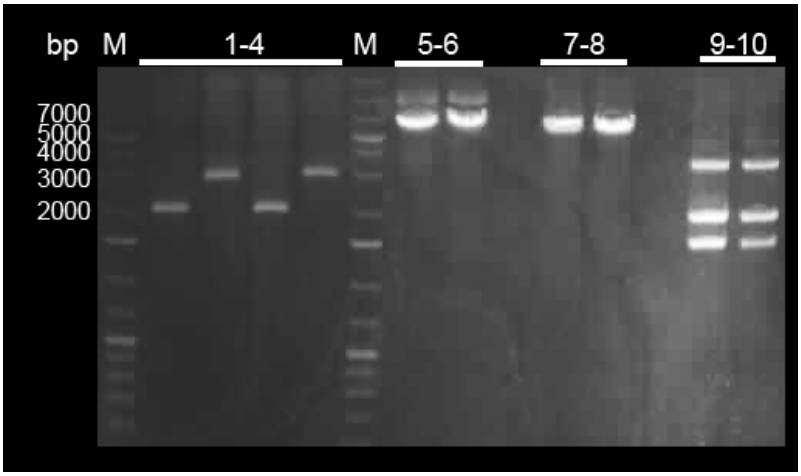


*Fig. 3.20 Gel-purification – **5-8**: mmoXYBZ (cut with Xba1 + Pst1); **9-10**: mmoDC+TT (cut with Xba1 + Pst1)*

Plasmid 181 is not showing the expected band length. It looks like, one of the restriction enzymes is not working correctly. Due to the fact, that the digestion of plasmid 182 is not showing a linear plasmid band, it is likely that XbaI is not working accordingly

Single test digestion of plasmid 178 with:
X, S, P in optimal buffer (3.1 / cutsmart) and X,S,P in buffer 2.1. Incubation in 37°C for 90 min.

1,0% agarose gel electrophoresis:

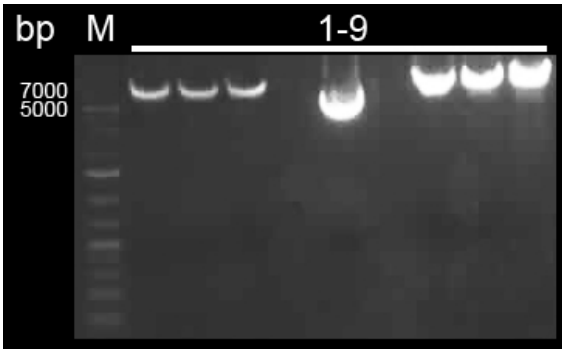


*Fig. 3.21 Test-restriction – **1**: mmoXYBZ (cut with Xba1 in Cutsmart buffer); **2**: mmoXYBZ (cut with Spe1 in Cutsmart buffer); **3**: mmoXYBZ (cut with Pst1 in buffer 3.1); **5**: uncut mmoXYBZ; **7**: mmoXYBZ (cut with Xba1 in buffer 2.1); **8**: mmoXYBZ (cut with Spe1 in buffer 2.1); **9**: mmoXYBZ (cut with Pst1 in buffer 2.1)*

→ all enzymes are working accordingly

Plasmid 178 (XYBZ) is digested with X+P and plasmid 179 (DC-TT) is digested with S+P. Both in buffer 2.1, to avoid buffer change. Incubation at 37°C for 90 min.

	XYBZ	DC-TT
Plasmid-DNA	5 µL	9 µL
Buffer 2.1	2 µL	2 µL
Enzyme I	1 µL	1 µL

Enzyme II	1 µL	1 µL
H_2O	11 µL	7 µL
∑	**20 µL**	**20 µL**

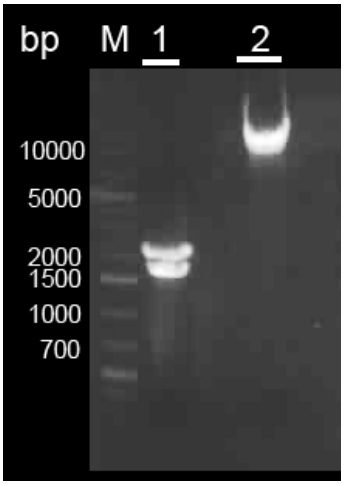


*Fig. 3.22 Gel-purification – **1**: mmoXYBZ (cut with Xba1 and Pst1); **2**: mmoDC + TT (cut with Spe1 and Pst1)*

→ the DC-TT samples is showing the right band length and is therefore cut out for further gel extraction

04.08.2014

Rüdiger, Oli

Gel extraction of DC-TT from 01.08.2014.

DNA concentration: first sample: 23.27 ng/µL ; second sample: 60,10 ng/µL

Restriction

of PL 178 (XYBZ) and of PL 179 (DC-TT)

PL 178 (XYBZ)	10 µL
2.1 Buffer	5 µL
Spe I	2 µL
Pst I	2 µL
H_2O	31 µL
∑	50 µL

PL 179 (DC-TT)	18 µL
2.1 Buffer	5 µL

Xba I	2 µL
Pst I	2 µL
H_2O	23 µL
∑	50 µL

-Incubation of restriction over night

05.08.2014

Rüdiger

Purification of PL 178 (XYBZ) cut wit S/P using Promega PCR Wizard

Purification of PL 179 (DC-TT) cut with X/P via gelelectrophoresis in small 1% agarose gel at 120 V

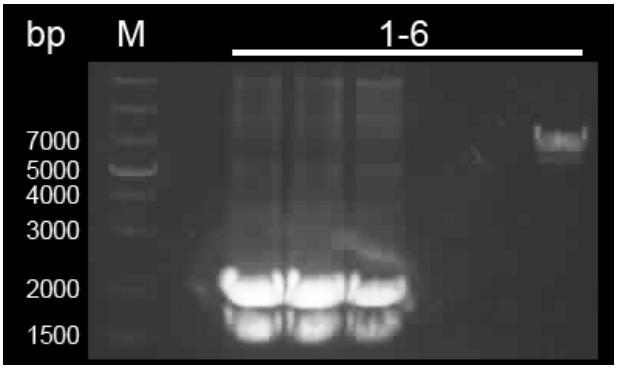


Fig. 3.23 *Gel-purification – 1-3: mmoDC + TT (cut with Xba1 and Pst1) (Exp. Length 1500 bp); 6: mmoXYBZ (cut with Spe1 + Pst1) (Exp. Length 5845 bp)*

→ restriction of XYBZ seems positive as well as DC-TT. The bands of DC-TT are cut out after 20 minutes more of elelectrophoresis of purificated using Promega PCR Wizard.

Ligation of XYBZ and DC-TT for 90 min at RT

Insert	14 µL
Vector	4 µL
T4 DNA Ligase	1 µL
T4 Ligase Buffer	2 µL
∑	**21 µL**

Transformation into competent E. coli cells and incubation over night at 37°C.

06.08.2014

Oli, Niels, Rüdiger

Colony-PCR

of 56 colonies

	Comment	**Volume per sample [µL]**	**Volume for X samples [µL]**
Template	Colony	0,25	16,25
5 x GoTaq-Puffer	contains NO Mg^{2+}	2	130
10 mM dNTPs		0,2	13
Primer 88	10 pmol μL^{-1}	0,5	32,5
Primer 89	10 pmol μL^{-1}	0,5	32,5
GoTaq Polymerase	5 U μL^{-1} (contains loading buffer)	0,05	3,25
MgCl		0,8	52
dH_2O		5,7	370,5
∑		10	650

Gelelctrophoresis in 1% agarose gel

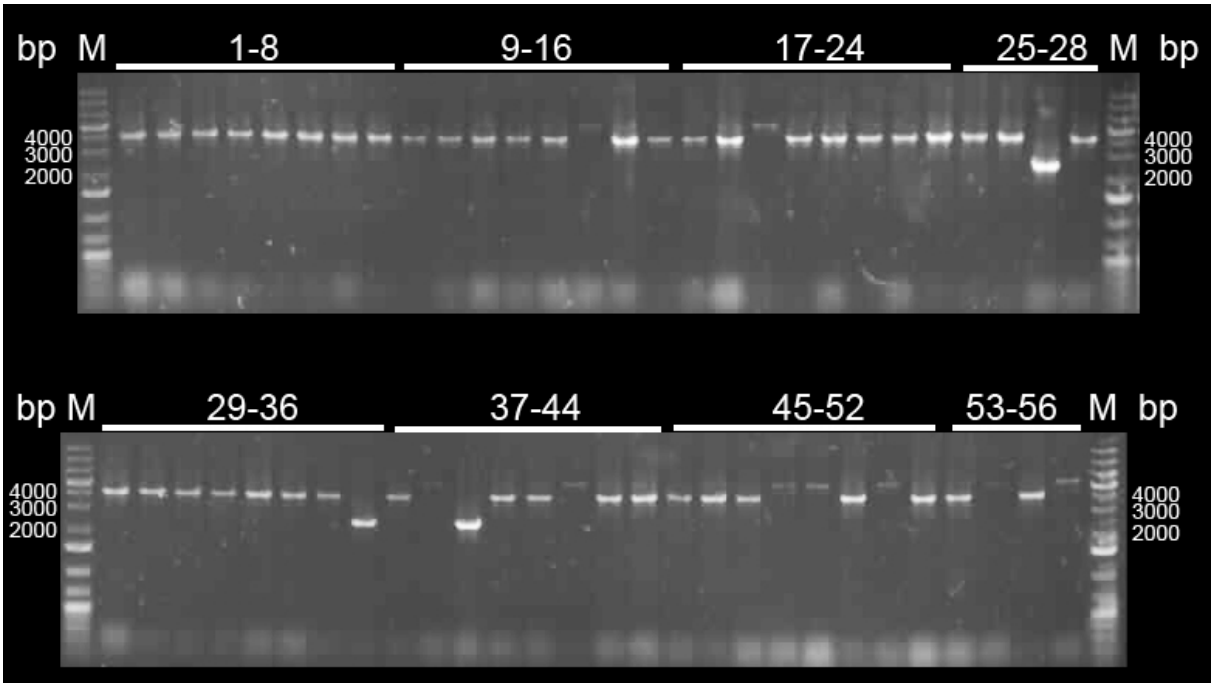


Fig. 3.24 Colony-PCR – 1-56: mmoXYBZDC+TT (Exp. Length 5600 bp)

→clones are positive: 14,19,38,42,48,49,51,54,56→ clones 19, 42, 56 are inoculated for overnight culture

→clones are negative: 1-13,15-18,20-37,39-41,43-47,50,52-53,55

07.08.2014

Steffen, Christian, Maren, Niels

Clones 19, 42, 56 restricted with X+P

Vectors: 84, 3 and 4 restricted with S+P

Restriction tested by use of gelelectrophoresis

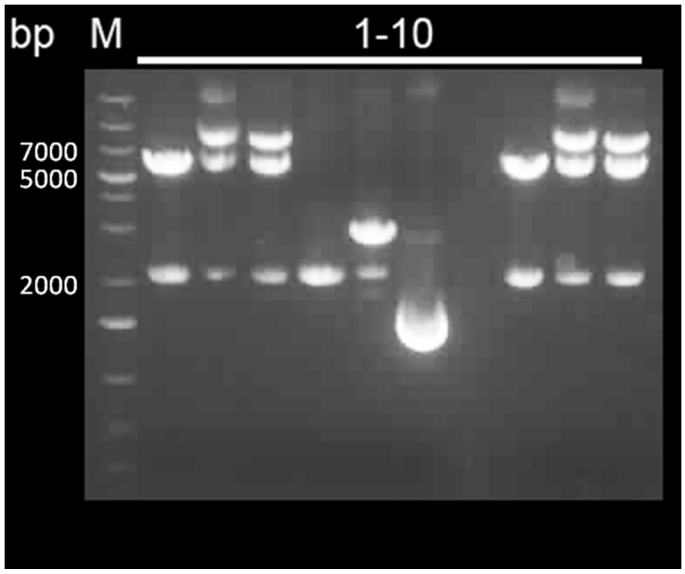


Fig. 3.25 *Test-restriction – **1**: mmoXYBZDC+TT (Clone 19); **2**: mmoXYBZDC+TT (Clone 42); **3**: mmoXYBZDC+TT (Clone 56); **4**: Lac-promotor [R0011] + RBS; **5**: Promotor [J23112]; **6**: Promotor [J23110]; **8**: mmoXYBZDC+TT (Clone 19); **9**: mmoXYBZDC+TT (Clone 42); **10**: mmoXYBZDC+TT (Clone 56) → 1-3+8-10 are cut with Xba1 and Pst1; 4-6 are cut with Spe1 + Pst1*

M, 19,42,56,84,3,4,empty, 19,42,56 (clone number (19,42,56) and plasmid numbers (84,3,4))

Conclusion: clones 19 and 84 are positive (expected Fragment size : 5300 bp, 2000bp)

Clone 19 was eluted

Clones 84 (lac-vektor) cleaned by use of promega kit

Ligation and transformation of clone 19 and 84 into xl1 blue mrf

08.08.2014

Niels, Maren, Oli

Colony PCR

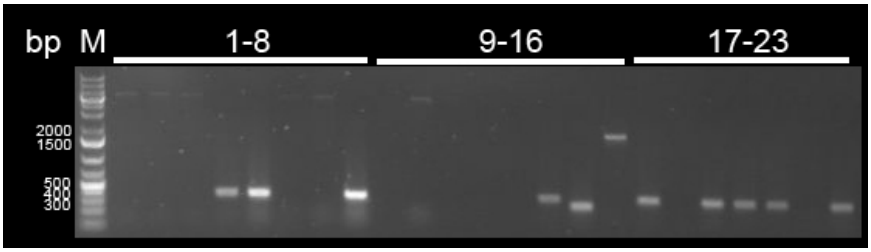


Fig. 3.26 Colony-PCR – 1-23: Lac-mmoXYBZDC-TT

→ positive clones: 1, 2, 3, 6, 7 and 9

10.08.2014

Oli

Inoculation of over night cultures of positive clones 1, 2 and 9.

11.08.2014

Steffen, Oli

Mini-prep of over night cultures.

Lac-XYBZDCTT clone 1, 2 and 9 are send for sequencing.

Restriction of Lac-End construct for ligation into new backbone: pSB1A3.
This backbone contains an ampicillin resistance and can be transferred into competent E. coli cells which were transformed with the chaperones (which are having a chloramphenicol resistance). On this way, we can check the effect of the chaperones on our construct.

pSB1A3	20µL
2.1 Buffer	3 µL
EcoRI	1 µL
PstI	1 µL
H_2O	5 µL
∑	**30 µL**

	Clone 1	Clone 2	Clone 9
Lac-XYBZDCTT	10µL	20µL	30µL
2.1 Buffer	2 µL	3 µL	4 µL
EcoRI	1 µL	1 µL	1 µL
PstI	1 µL	1 µL	1 µL
H_2O	6 µL	5 µL	4 µL
∑	**20 µL**	**30 µL**	**40 µL**

Ligation of Lac-XYBZDCTT in pSB1A3 and plating on amp-plates with and without glucose.

12.08.2014

Oli, Carsten

Growth is observed on both plates.

In order to screen for positive clones, colony-PCR of 40 clones is performed.

Gelelectrophoresis

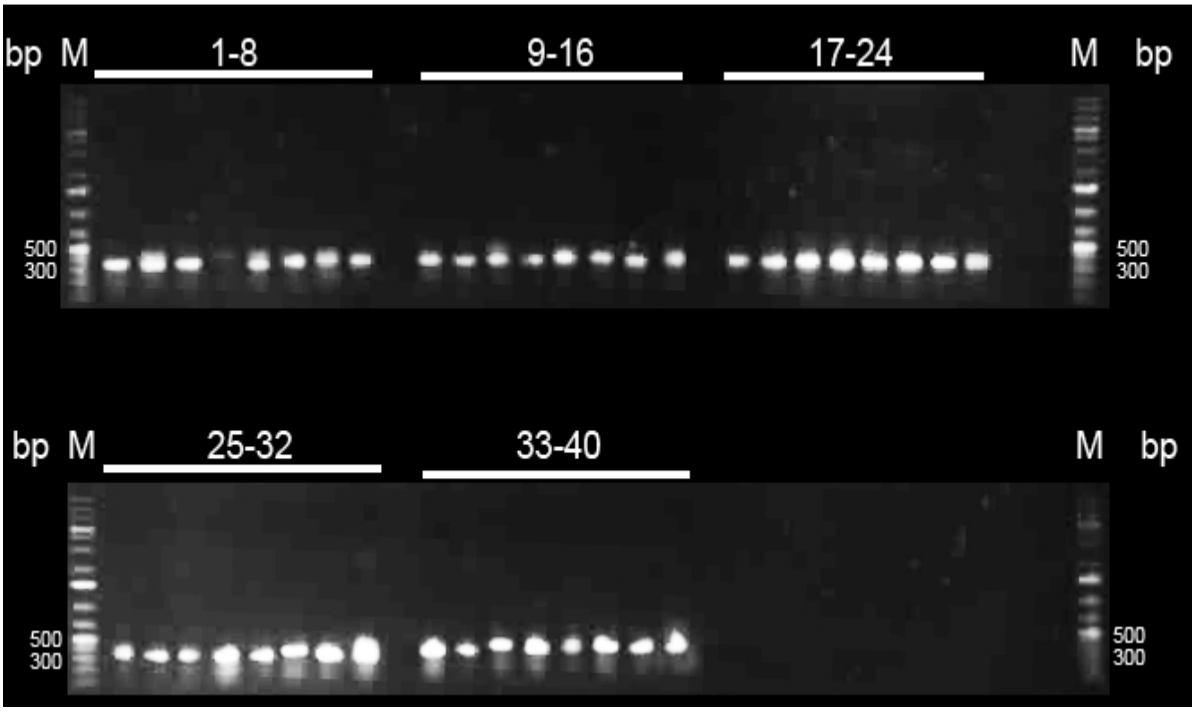


*Fig. 10.27 Colony-PCR – **1-40**: Lac+XYBZDC+TT in pSB1A3*

→ no positive clones could be observed→ colony PCR is repeated the next day

Ligation

of Lac+final construct and pSB1A3 is repeated over night at 16 °C

13.08.2014

Maren, Lukas, Rüdiger

Colony-PCR

Colony PCR of construct Lac+XYBZDCTT is repeated with 40 clones from the plates from 13.08. Extension time 6min30s.

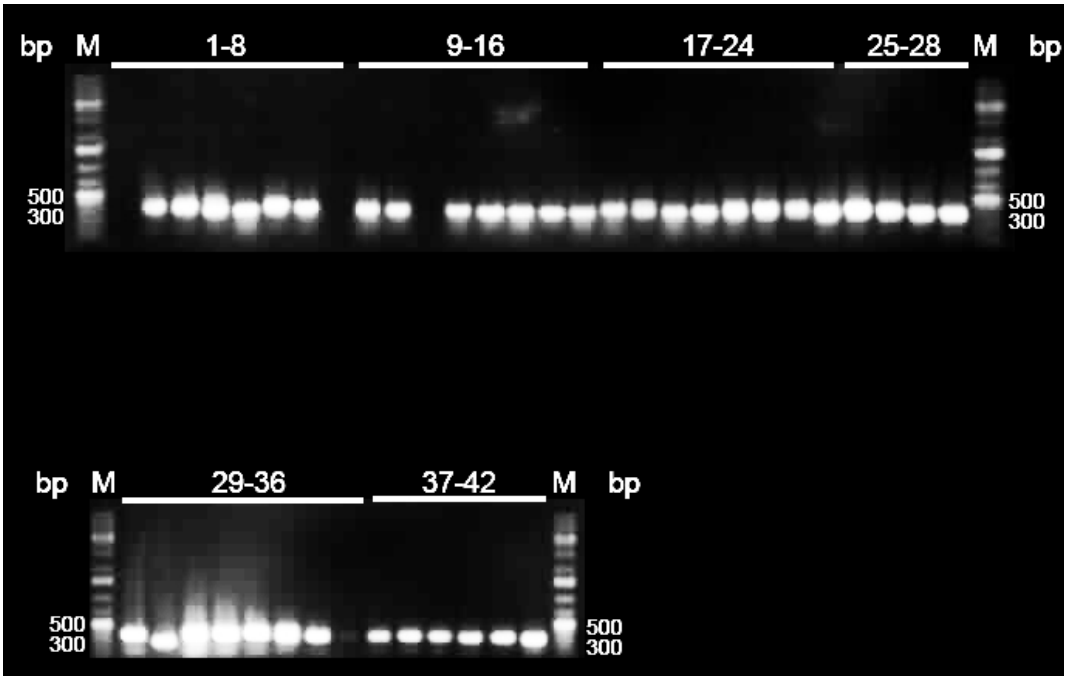


Fig. 3.28 Lac+XYBZDCTT in pSB1A3 (Exp. Length 6000) M:1kB plus ladder

→No positive colonies could be observed.

Transformation

Of the over night ligation of Lac+final construct and pSB1A3.

14.08.2014

Oli, Anna, Rüdiger

Colony PCR

Of 30 clones of the transformation from 14.08. of Lac+final construct and pSB1A3 (Extension time 6min 30s, Primer 88/89).

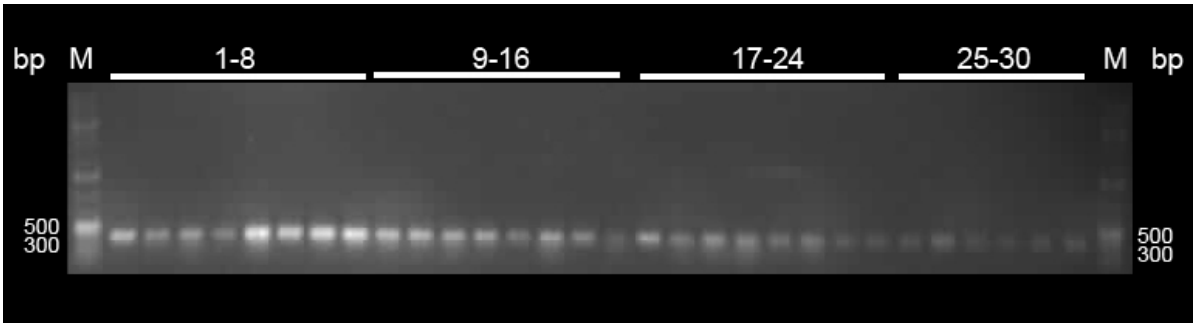


***Fig. 3.29** Repetition of Colony-PCR – **1-30**: Lac+mmoXYBZDC+TT in pSB1A3*

→ All clones are negative

15.08.2014

Niels, Steffen, Oli

PCR of End-construct with “promotor-primer” in order combine J23100 and J23112 with the end-construct. Primer used: 125/126 (containing the promotor sequence and binding to mmoX) and 89 (suffix). Polymerase: pfu-polymerase

DNA: 10 ng of plasmid 194 (iGEM2014-10-mmo-XYBZ-DC-TT Klon 19).

18.08.2014

Maren, Zen Zen, Niels, Steffen, Rüdiger

Restriction

Restriction of the Backbone J61002 (PL 201) with EcoRI and PstI for obtaining material for changing the resistance of end-construct and end-construct+ strong/weak constitutive promoter (J23101, J23115) to ampicillin.

Fragment:	15 µL (=5 µg)
Cut-Smart Buffer	2 µL
EcoRI-HF	3 µL
H_2O	0 µL
∑	**20 µL (incubation for 1h at 37°C)**
+ H_2O	5 µL
+ PstI	2 µL
+ Puffer 3.1	3 µL
∑	**30 µL (incubation for 3h at 37°C)**

- After incubation 1 µL Antartic Phosphatase was added and incubated for 30 min.

-Clean up via Promega PCR Wizard

Additionally, the Lac+end (PL 204) construct is restricted with EcoRI and PstI for cloning into the backbone.

Fragment Lac+end PL 204	15 µL (~5 µg)
Cut-Smart Buffer	2 µL
EcoRI-HF	0,5 µL
H_2O	2,5 µL
∑	**20 µL (incubation for 1h at 37°C)**
+ H_2O	6,5 µL
+ PstI	0,5 µL
+ Puffer 3.1	3 µL
∑	**30 µL (incubation for 1h at 37°C)**

-Clean up via gel electrophoresis

*Fig. 3.30 Gel-Purification - **1-3**: Lac+mmoXYBZDC+TT cut with EcoR1 and Spe1*

→ upper bands were cut out and eluted with Promega Gel Wizard

Ligation

Lac+end and backbone J61002 are ligated

Insert (Lac+end cut with E/P)	7 µL
Vector (J61102)	2 µL
T4 DNA Ligase	0,5 µL
T4 Ligase Buffer	1 µL
∑	10,5 µL

- ligation over night at RT

Test of PCR Products

Gelectrophoresis of PCR products end-construct+ strong/weak constitutive promoter (J23100, J23112)

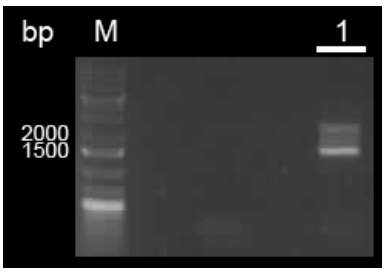


Fig. 3.31 Promotor Attachment PCR – 1: Promotor J23112 + mmoXYBZDC+TT (Exp. Length 5300 bp)

→ PCR products show the wrong length, PCR is therefore repeated.

PCR

PCR for combining promoters with end-construct is repeated with Phusion polymerase and more template DNA

	Kommentar	**Menge pro Ansatz [µL]**
Template (PL 194)	~1 ng μL^{-1}	0,7 (=20 ng)
5 x Phusion HF Buffer	enthält Mg^{2+}	10
10 mM dNTPs		1
Primer 125/126	10 pmol μL^{-1}	2,5
Primer 89	10 pmol μL^{-1}	2,5
Phusion DNA Polymerase	2 U μL^{-1}	0,5
dH_2O		32,8
$\sum$		50

Maren, Niels, Rüdiger

19.08.2014

Gelelectrophoresis

For testing, whether the repeat of PCR from 18.08.2014 worked

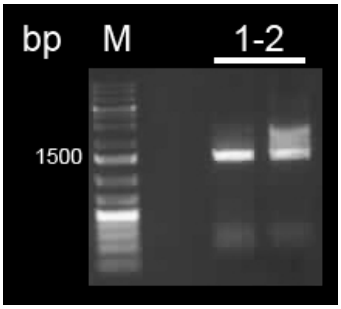


*Fig. 3.32 Promotor Attachment PCR – **1**: Promotor J23110 + mmoXYBZDC+TT (Exp. Length 5300 bp); **2**: Promotor J23112 + mmoXYBZDC+TT (Exp. Length 5300 bp)*

→ Did not work. PCR products show the wrong length again. We suspect that the annealing is unspecific, because the RBS sequence, that the forward primer also binds to is in between every gene. PCR is therefore repeated with higher annealing temperature to increase specificity.

Gradient PCR

Annealing temperature range: 60 – 70 °C

Six samples per promoter were prepared.

	Kommentar	**Menge pro Ansatz [µL]**	**Menge f. X Ansätze [µL]**
Template	~1 ng μL^{-1}	~10 ng	0,5
5 x Phusion HF Buffer	enthält Mg^{2+}	10	130
10 mM dNTPs		1	13
Primer 89	10 pmol μL^{-1}	2,5	32,5
Primer 125/126	10 pmol μL^{-1}	2,5	16,25/16,25

Phusion DNA Polymerase	2 U μL^{-1}	0,5	6,5
dH_2O		33,5	435
∑		50	650

-Gelelectrophoresis to test, whether the PCR products show the expected length

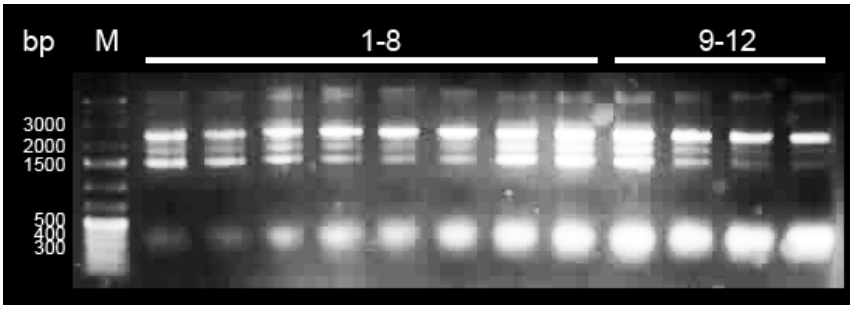


*Fig. 3.33 Gradient-PCR for promotor attachment with temperature range 60-70 °C – **1-6**: J23112+mmoXYBZDC+TT (Exp. Length 5300 bp); **7-12**: J23110+mmoXYBZDC+TT (Exp. Length 5300 bp)*

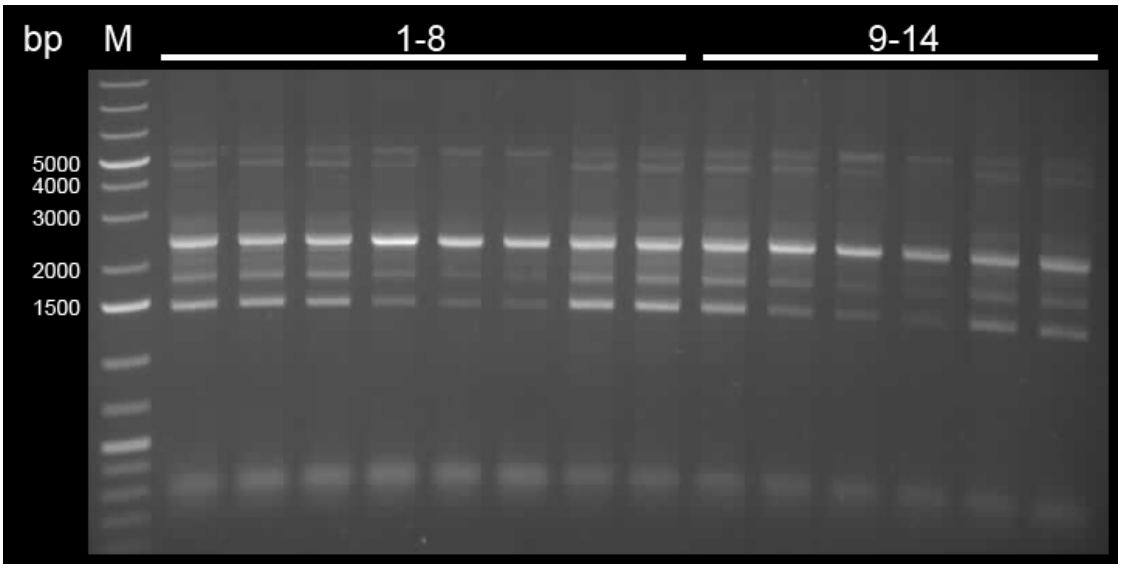


Fig. 10.34 Repetition of PCR for promotor attachment in GTO agarose – 1-6: J23112+mmoXYBZDC+TT (Exp. Length 5300 bp); 7-12: J23110+mmoXYBZDC+TT (Exp. Length 5300 bp)

→ There are multiple PCR products. At around 5000 bp a weak band of a product can be seen. This should be the product that is wanted. The entire PCR samples are therefore run in another agarose gel, and the bands with the right length are cut out.

→ the upper bands were cut out, the most right was forgotten

Transformation

Via heatshock of the ligation reaction Lac-end + J661002 (Amp) that was run over night.

-100 mL and rest are plated and cultivated over night

Maren, Niels, Rüdiger

20.08.2014

Colony-PCR

Colony-PCR of 30 colonies of Lac-end + J661002 clones (Amp) (19.8.14) with Go-Taq protocol.

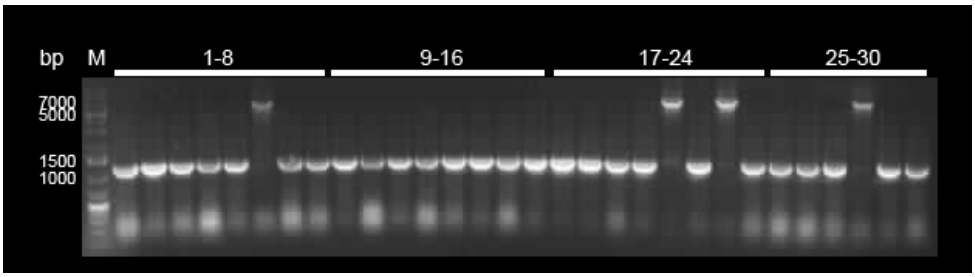
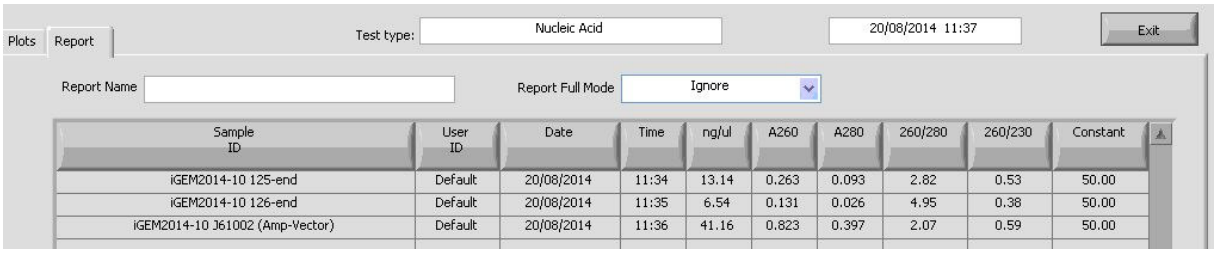


Fig. 3.35 *Colony-PCR – 1-30: Lac-mmoXYBZDC+TT in J661002*

Clone 6, 21, 23 and 28 are positive. The four clones were inoculated in 2YT Amp over night.

Gelelution

With Promega PCR Wizard



Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant
iGEM2014-10 125-end	Default	20/08/2014	11:34	13.14	0.263	0.093	2.82	0.53	50.00
iGEM2014-10 126-end	Default	20/08/2014	11:35	6.54	0.131	0.026	4.95	0.38	50.00
iGEM2014-10 J61002 (Amp-Vector)	Default	20/08/2014	11:36	41.16	0.823	0.397	2.07	0.59	50.00

→ concentrations are very low and not sufficient for further cloning, therefore another PCR is set up with a specific prefix primer, that binds to the sequence of primer 125/126, so more product can be retrieved.

PCR

on the PCR product that was cut out on 19.08.2014

Annealing temperature: 65 °C

	Kommentar	**Menge pro Ansatz [µL]**	**Menge f. X Ansätze [µL]**
Template 125-end/126-end	~1 ng μL^{-1}	1	2/1
5 x Phusion HF Buffer	enthält Mg^{2+}	10	20
10 mM dNTPs		1	2
Primer 89	10 pmol μL^{-1}	2,5	5
Primer 2041 (prefix primer)	10 pmol μL^{-1}	2,5	5

Phusion DNA Polymerase	2 U μL^{-1}	0,5	1
dH_2O		32,5	64
∑		50	100

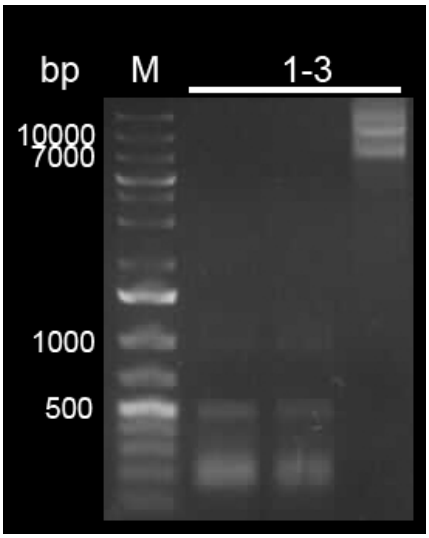


Fig. 3.36 PCR M 1:J23112- mmoXYBZDC-TT (Exp. Length: ≈ 5300 bp); 2: J23100-mmoXYBZDC-TT (Exp. Length: ≈ 5300 bp); 3: Lac-mmoXYBZ DC-TT as positive control

→ The PCR did not work as intended.

21.08.2014

Oli, Maren

Plasmid preparation and GS of 4 positive clones of Lac-End in pSB1A3. Clone 6, 21, 23 and 28.

These clones will be used for further expression of the sMMO.